

THE APPLICATION OF DENSITY GRADIENT CENTRIFUGATION TO PALYNOLOGY

R.M. FORSTER

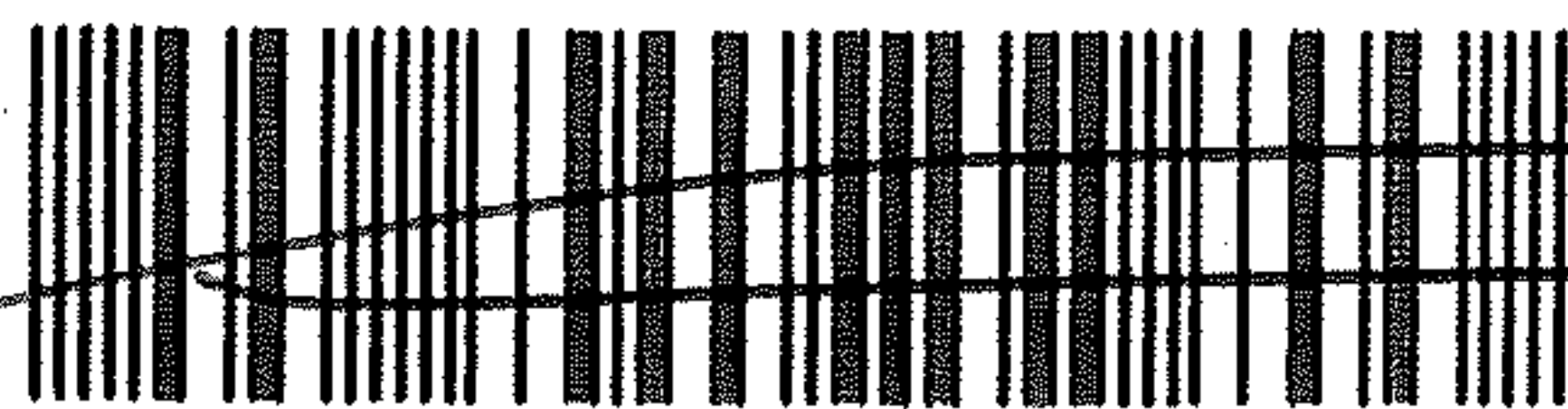
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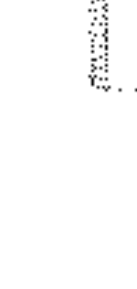
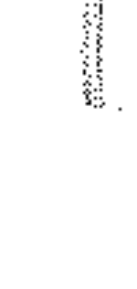
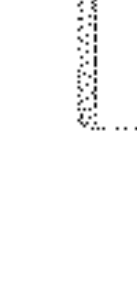
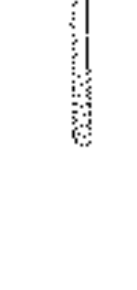
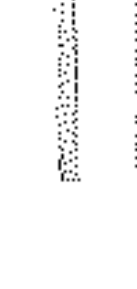
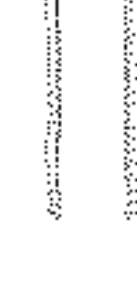
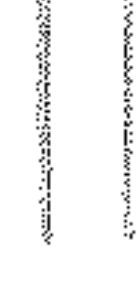
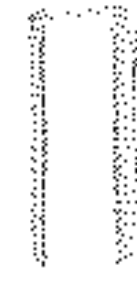
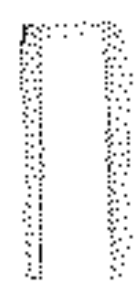
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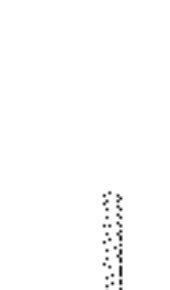
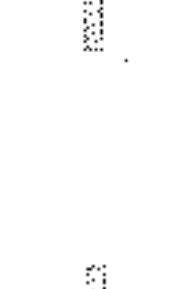
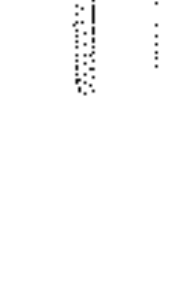
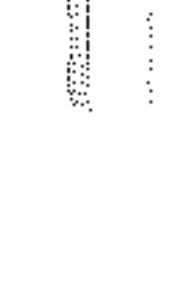
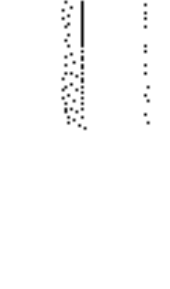
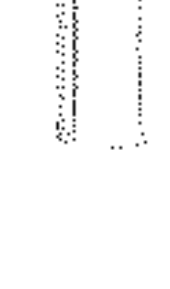
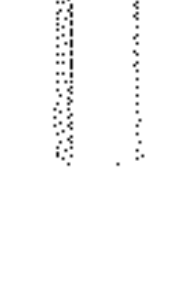
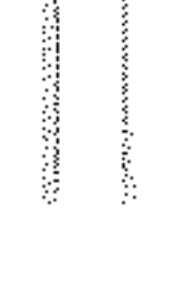
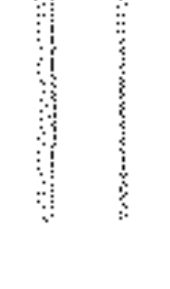
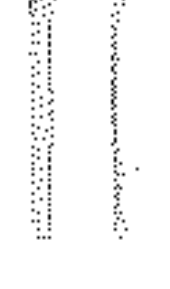
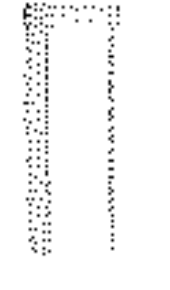
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INTRODUCTION

Density (used here as synonymous with specific gravity) is a very important property for particles subjected to sedimentary processes. It is prominent in Stokes' Law, which controls deposition rate, and many important industrial separations (e.g. concentration of alluvial tin) depend entirely upon it. These facts make it all the more surprising that so little attention has been paid to the density of pollen and spores.

There have, however, been several measurements of the density of whole pollen grains (e.g. Gregory, 1961) and Wolfson (1954) used flotation to measure this parameter for the pollen exine. Flenley (1971) used the previously untried technique of density gradient centrifugation (Brakke, 1960; Hermans and Ende, 1964). He found that different species of plants produced exines with slightly different densities.

It therefore seemed worthwhile to follow up this work, using improved gradient techniques to investigate several matters. In the first place it was desirable to measure the density of more pollen types as accurately as possible. It was also necessary to find whether these differences were maintained in the fossil condition and with various chemical treatments. There was always the faint possibility that density might prove to be one of the parameters useful in separating different pollen groups for machine counting, thus leading towards the automation of palynology. A final and perhaps more realistic hope was that pollen exines might prove to differ significantly in density from other insoluble fossil organic matter. A separation of pollen from organic detritus would be a breakthrough in fossil pollen preparation, and is probably a necessary step to automation of palynology by pattern recognition, a procedure now showing some prospect of being achieved (Langford *et al.*, 1986).

This publication is a preliminary report on the stage so far reached in these investigations. A fuller account is given in Forster (1986).

DEVELOPMENT OF METHODS

The methods which have been developed are based on those of Flenley (1971), with modifications.

i) Gradient materials

The gradient components which were used in preliminary experiments, propanone and 1,2 dibromoethane, are not ideal liquids for density gradient work; both are highly volatile making mixtures susceptible to density drift when not completely sealed. In addition, 1,2-dibromoethane is toxic by inhalation, harmful when in contact with the skin or eyes and is a recognised carcinogen. A suitable replacement has been found in

potassium iodide (KI), solutions of which possess many of the properties which an ideal gradient material should have (Pertoft and Laurent, 1969), at least for use with isolated pollen exines which do not contain living protoplasts. For example, using KI, solutions of high density and low viscosity can be formed; a saturated solution at 12°C has a density of about 1.697 g cm⁻³ and, between c. 35 and 55% w/w concentration (the range giving the required densities), the viscosity of any KI solution is close to that of water at the same temperature.¹ KI does not react noticeably with pollen exines, it does not present a major health hazard and it is not prohibitively expensive, being only a fraction of the cost of caesium chloride, a salt commonly used in gradient work for very high-density, aqueous solutions. Another possible advantage of KI is the monovalency of its ions; the Schulze-Hardy rule states that the tendency of an electrolyte to flocculate small, lyophobic particles is greatly dependent upon the valency of the counter-ions though almost independent of the character of the various ions, the valency of the co-ions and the concentration of the particles. Monovalency is, therefore, advantageous.

ii) **Temperature control**

Most investigations which have utilised density gradient centrifugation as a density measuring tool have been performed at constant temperature (e.g. Shortman (1969), Kozinski and Szbalski (1959)). The accurate measurement of polythene density using gradients of ethanol (which has a rate of change of density with temperature similar to that of 50% w/w KI solution) is considered to require temperature control to $\pm 0.1^\circ\text{C}$ (Christy, P.F., Imperial Chemical Industries, personal communication). For the purpose of exine density measurement all operations are performed with an ambient temperature of 12°C and a mean deviation from this temperature of 0.5°C.

iii) **Gradient formation**

The gradient generator (Fig. 1) is based on that of Flenley (1971) but with several important differences. The mixing of 'light' and 'heavy', components is achieved by magnetic stirrer. The gradient solution is pumped from the mixing chamber at a constant and reproducible rate by peristaltic pump rather than being gravity fed and hand-regulated. The least dense portion of the gradient is formed first, liquid of increasing density being introduced at the bottom of the centrifuge tube where it displaces upward the liquid already there. This eliminates the need to trickle a thin stream of liquid down the side of the centrifuge tube, a procedure which could allow loss of solvent by evaporation. These changes in technique favour improved gradient linearity and reproducibility. With a pumping rate of c. 20 ml hr⁻¹ a gradient may be formed in a 13 ml glass centrifuge tube in approximately 30 minutes. If a heavy-light component difference of 0.14 g cm⁻³ is used, reasonably linear density gradients of between 0.017 and 0.019 g⁻³cm⁻¹ can be reproduced.

¹ Data from International Critical Tables.

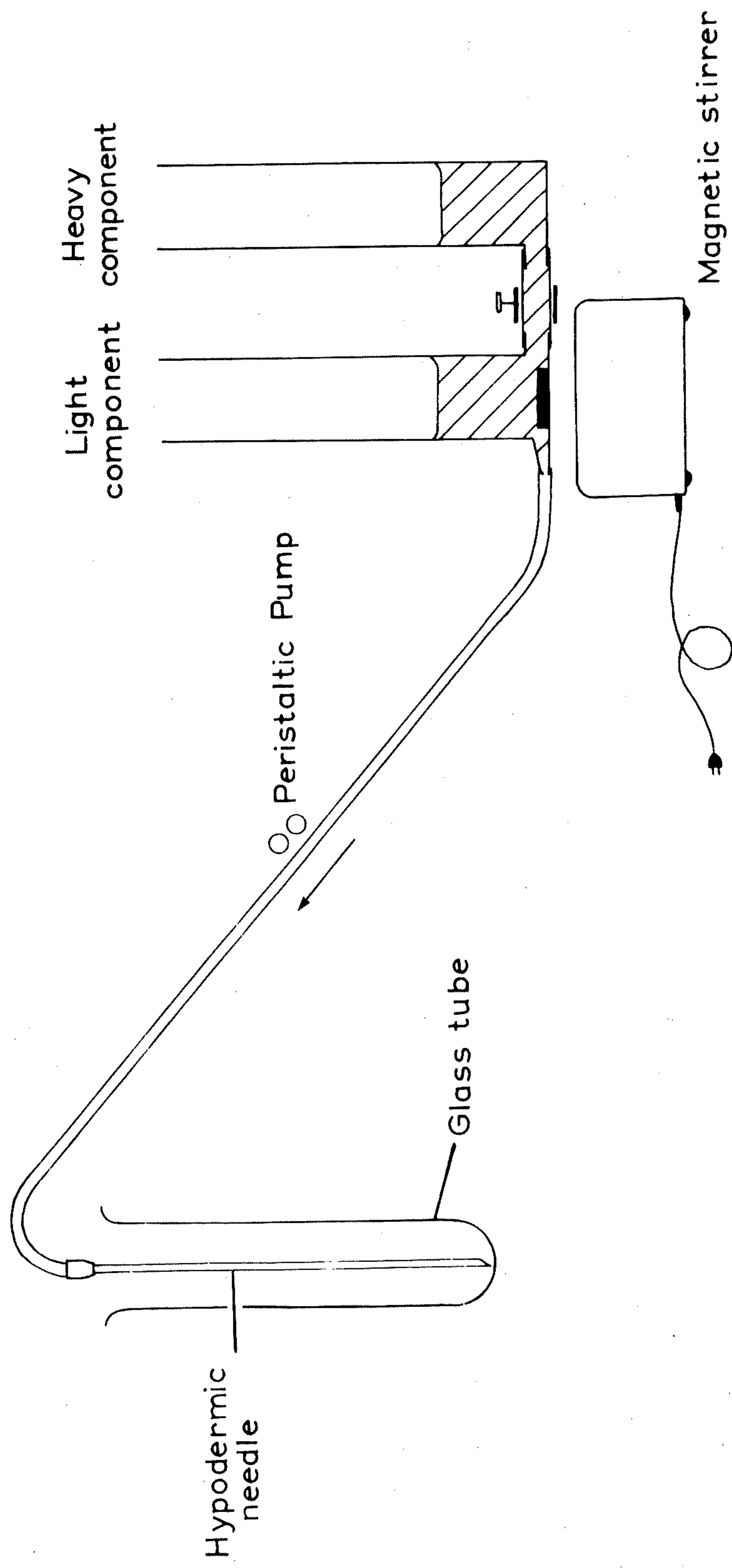


Figure 1 Gradient forming apparatus.

iv) **Sample preparation and centrifugation**

Fossil samples for use with gradients are prepared by standard palynological techniques, the aim being to purify and concentrate the fossil palynomorphs as much as possible and to remove any intine remnants. To this end any combination of the following may be used: potassium hydroxide digestion, acetolysis, hydrofluoric acid and hydrochloric acid dissolution, sodium chlorate oxidation and sieving (including removal of clay and fine silt by ultrasonic sieving).

Modern pollen samples for density measurement are prepared by removing protoplast and intine with potassium hydroxide and acetolysis treatments. A degree of standardisation is effected by maintaining a pollen concentration of about 100 ml reagent per gram of air dried pollen during treatment and using an acetolysis mixture of 9:2, concentrated sulphuric acid: acetic anhydride for all experiments. To ensure the complete removal of intine and protoplast, acetolysis is allowed to proceed at 100°C for 30 minutes. After preparation the exines are stored in distilled water for at least 100 hours to allow density equilibration (see results section).

Prior to centrifugation sample is applied to the gradient surface. In the case of modern pollen for density measurement, between 3×10^5 and 3.6×10^5 exines of the type to be measured in 0.2 ml of suspension are floated onto the top of the gradient; this volume of suspension conforms to the guidelines for gradient loading given by Williams and Wilson (1981). The sealed gradient is spun in a bench top centrifuge, with swing-out head, until equilibrium is achieved. Because a refrigerated or thermostatic centrifuge is not available centrifugation time is kept to a minimum in order to reduce heating and consequent interference with accurate density measurement, where this is desired. Calculations based upon the equation derived by Thomson and Mikuta (1954, 1955 (erratum)) from an approximation of Stokes' law and a modification of this equation which takes some account of the hollow, perforated nature of the pollen exine, suggest that, in the centrifuge and with the density gradients normally used, 15 to 20 minutes at 3800 r.p.m. should be sufficient to bring exines of relatively small diameter to within 1µm of equilibrium.

v) **Fractionation**

The gradient is withdrawn by peristaltic pump from the bottom of the glass centrifuge tube through a hypodermic needle lowered carefully into the gradient. About thirty fractions are collected manually by drop counting in 4 ml sample tubes.

vi) **Density determination (modern pollen types)**

The density of a fraction is determined by weighing a known volume dispensed from a 50 µl syringe of the type used for gas chromatography. Using a balance sensitive to 0.1 mg the mean of 10 weighings per fraction is taken. The coefficient of variation of 10 such weighings is less than 0.3%. The densities of several fractions from the central, linear part of the gradient containing the pollen band are measured and a least squares regression

line of density on fraction number is fitted. This can be achieved with a mean residual of $<1 \times 10^{-3} \text{ gcm}^{-3}$. The exine concentration in each fraction is determined by haemocytometer (improved Neubauer for small types, modified Fuchs-Rosenthal for larger exines; only the fractions containing $>2\%$ of the peak fraction concentration are counted because of the tendency for some pollen to stick to the sides of the centrifuge tube during centrifugation and to fall away during fractionation. This results in almost all fractions above the pollen band containing occasional, stray exines. The mean fraction number is computed and converted to a mean density value using the regression line.

vii) Use of the Coulter Counter (model ZB) for automatic counting

Problems have been encountered in the application of this instrument. Firstly, it is doubtful whether sufficiently large numbers of palynomorphs, fossil or otherwise, can be recovered in the fractions from even a large density gradient for counting by this method; the Coulter Counter requires fairly large volumes of suspension on which to work - at least 150 ml for a reasonable size distribution. Secondly the Coulter Counter does not appear to treat acetolysed exines as solid particles; the presence of electrolyte in the exine interior and the apertures leading to it influences size measurements. The result is a considerable reduction in the modal size and standard deviation of size obtained for a sample of any pollen type. This is illustrated in Fig. 2 which shows the size distributions obtained for a sample of air dried *Corylus avellana* pollen and a sample of the same after acetolysis to remove protoplasts. The implications of this phenomenon are twofold. Firstly, the ability of the counter to distinguish between different types of palynomorph on the basis of size may be much reduced; there will be a loss of resolution. Secondly, in the mode necessary for the sizing or counting of most exines, counts of particles which register sizes of less than 2 or 3 μm may be subject to interference, usually in the form of excessively high counts; this may make counts of some exine types unreliable.

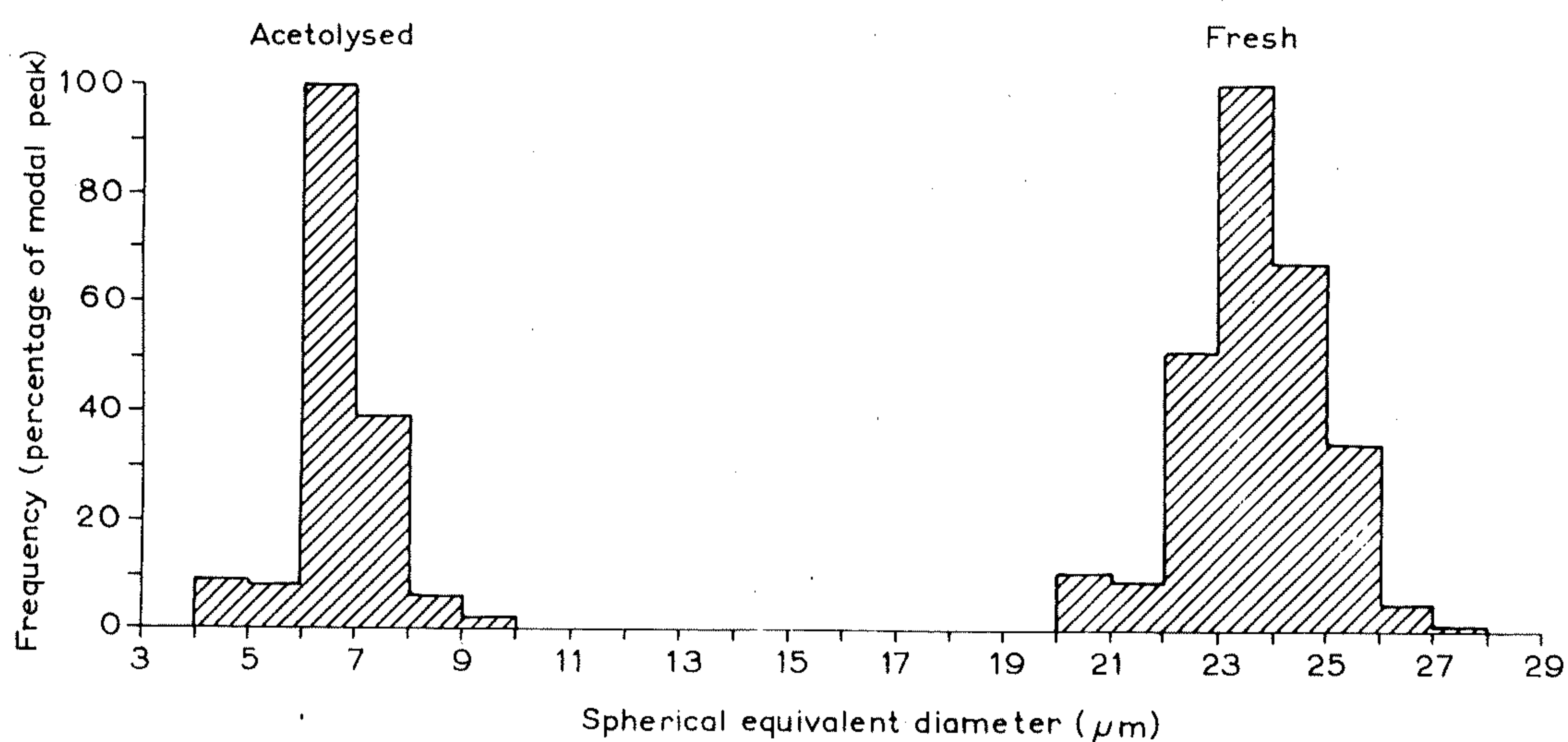


Fig. 2 Sizing of *Corylus avellana* exines by Coulter Counter.

viii) **Rate-zonal centrifugation**

Calculations suggest that, because of the relatively large size of most pollen exines and their consequent rapid sedimentation rate, rate-zonal separation on a density gradient may be difficult. However, much depends upon the manner in which they sediment - as hollow spheres, solid spheres or as intermediate forms. No serious attempt has been made to apply this technique to the separation of different exine types though it may be possible to find a suitable combination of gradient and centrifugation conditions.

SUMMARY OF RESULTS AND DATA

- a.) Clean mixtures of several modern exine types have been separated routinely. Fig. 3 illustrates in graph form the result of separating a mixture of *Dactylis glomerata*, *Corylus avellana* and *Betula spp.* on a density gradient. Plate 1a is a micrograph of the original pollen mixture. Plates 1.b,c, and d show the contents of the *Dactylis*, *Corylus* and *Betula* bands respectively.
- b.) Separations of certain optically indistinguishable taxa (Gramineae sp. and *Corylus avellana/Myrica gale*) have not been possible with equilibrium density gradient centrifugation. This is, perhaps, not surprising as optically similar species tend to be taxonomically similar.
- c.) It has proved possible to measure exine density accurately using the methods described previously. Fig. 4 is an example of the type of data from which mean density is calculated. Reproducible figures are only possible when density has been allowed to equilibrate, in distilled water, after preparation of the exine. Measurements made on *Corylus avellana* have shown that after preparation its density increases by more than $1.3 \times 10^{-2} \text{ gcm}^{-3}$ before stabilising after 80-100 hours (Fig. 5). More approximate measurements on *Dactylis glomerata* and *Betula sp.* suggests that changes in density of a similar order occur in these species. Thus, providing that exines are stored in water for a suitable period prior to analysis, reproducible results may be obtained; the four stable measurements of *Corylus* mean density which appear in Fig. 5 have a range of only $1.4 \times 10^{-4} \text{ gcm}^{-3}$ (i.e. $\pm 7.0 \times 10^{-4} \text{ gcm}^{-3}$ or c. 0.05% of their mean.)
- d.) To date, the densities of five exine types have been accurately measured:

<i>Dactylis glomerata</i>	1.4737
<i>Ulmus sp.</i>	1.4988
<i>Corylus avellana</i>	1.5064
<i>Quercus sp.</i>	1.5370
<i>Betula sp.</i>	1.5552

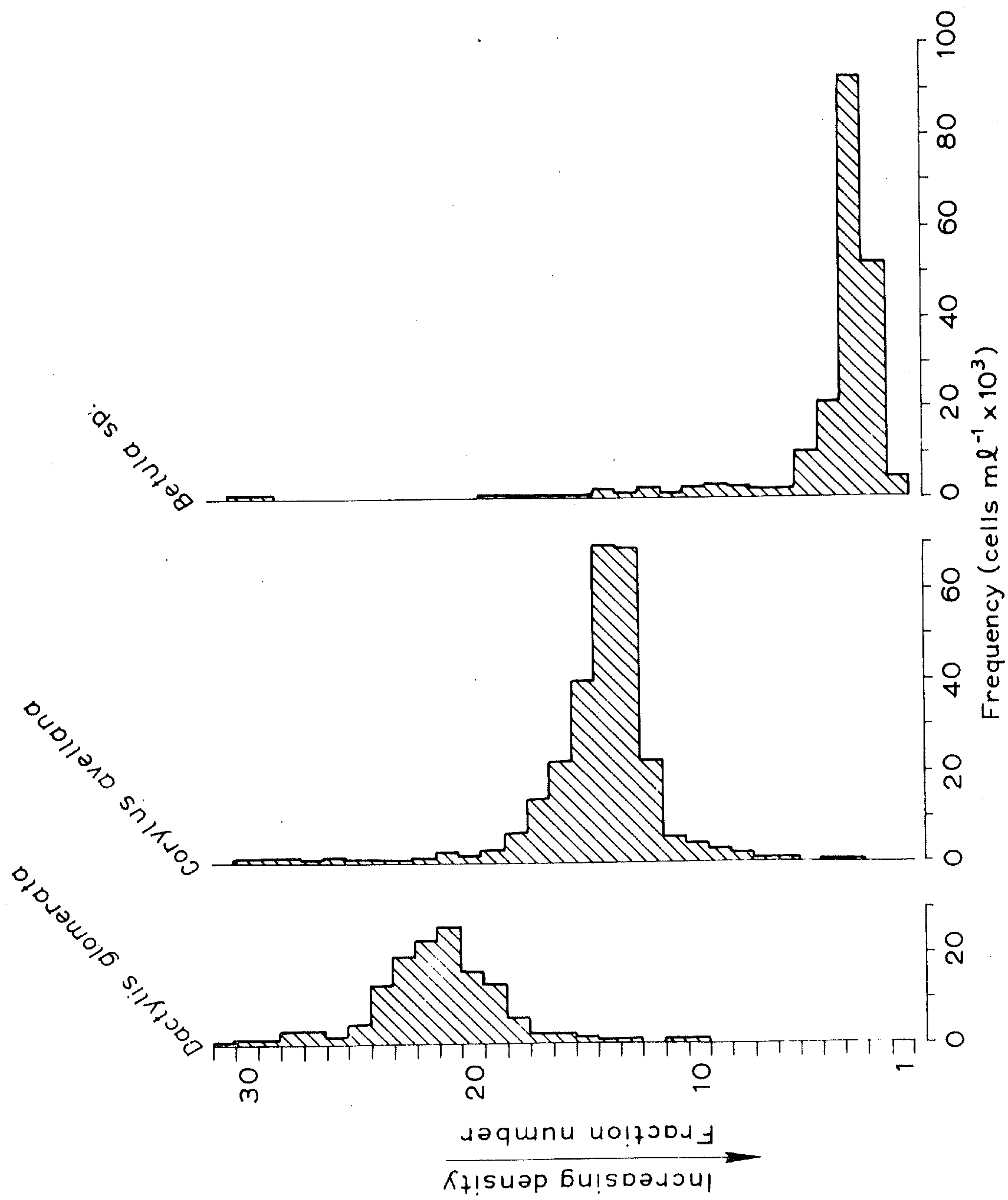


Figure 3 Separation of 3 modern pollen types on a density gradient.

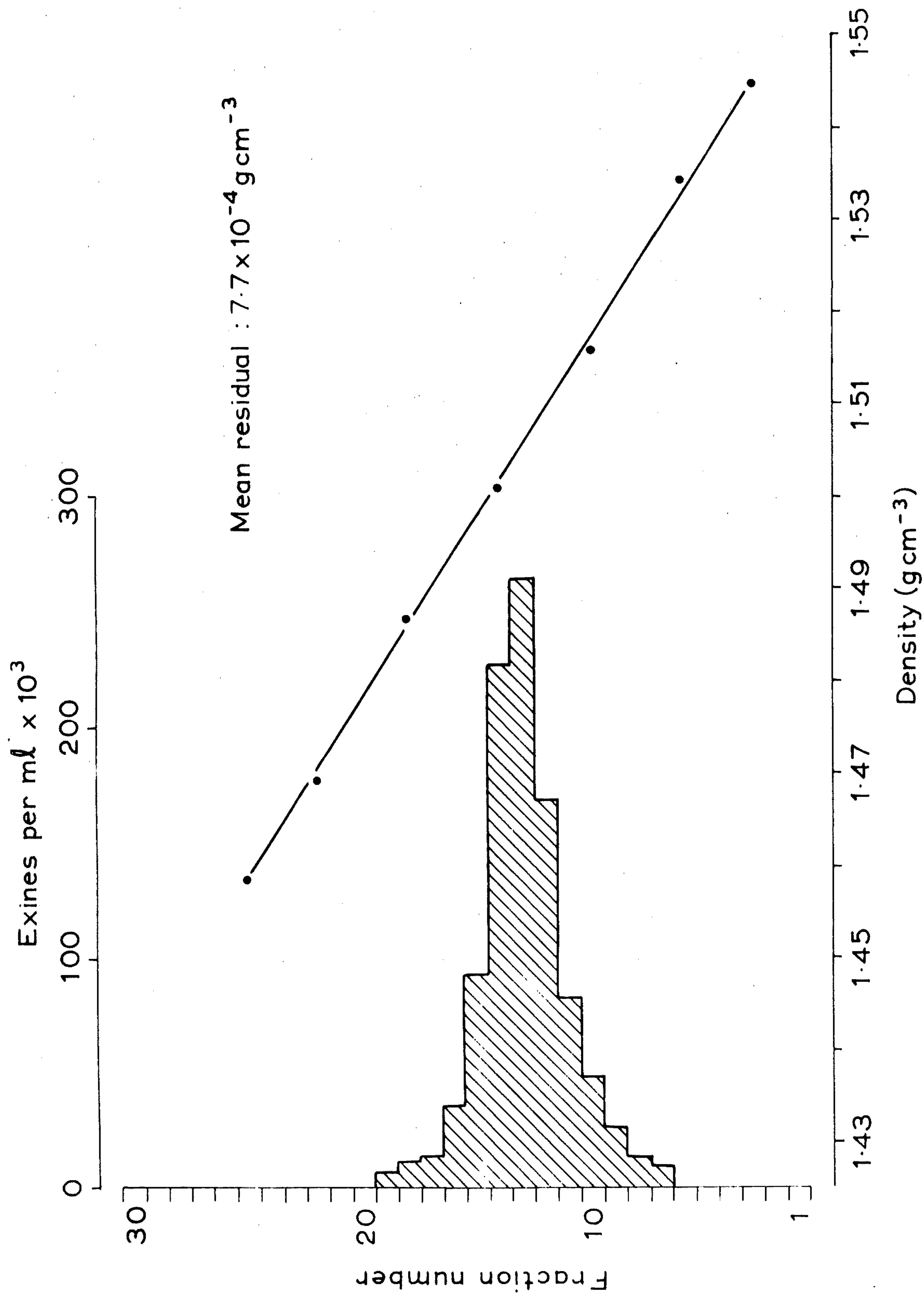


Figure 4 Density data from a sample of *Corylus avellana* centrifuged to equilibrium.

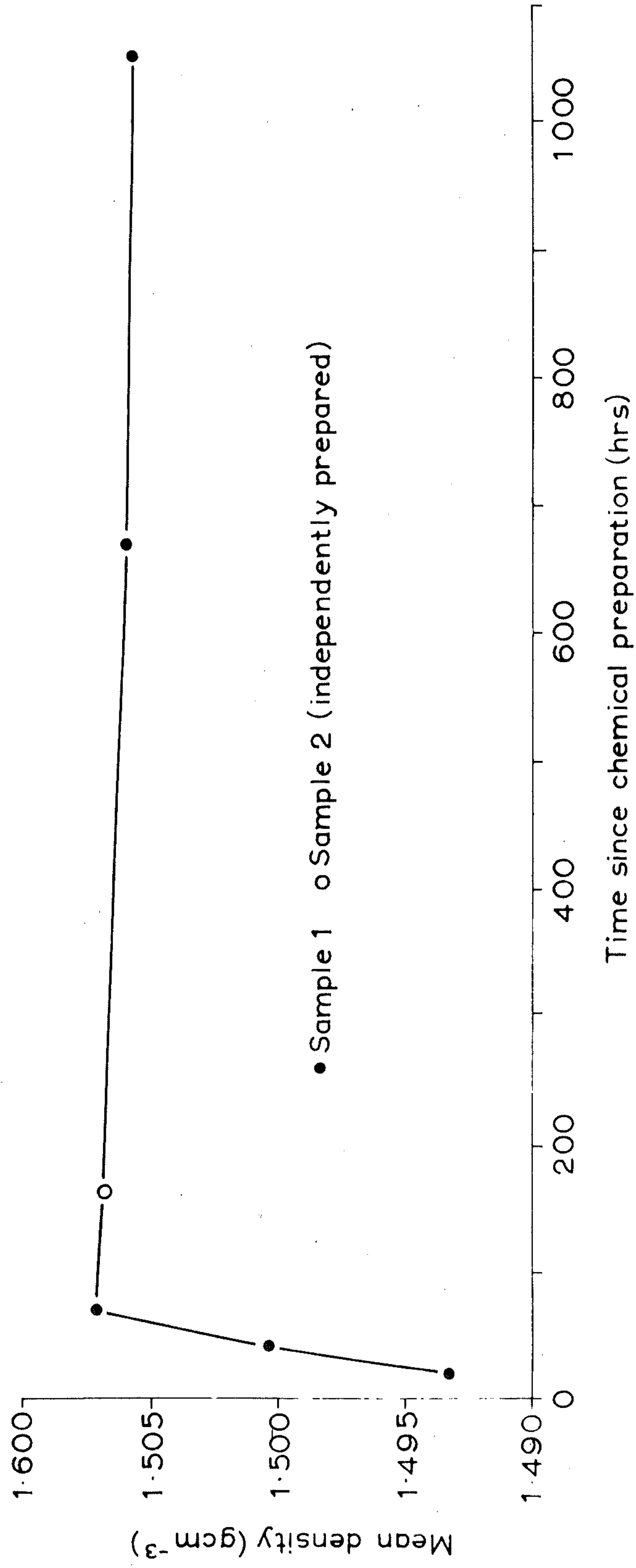


Figure 5 Change in the density of *Corylus avellana* exines stored in water after chemical preparation.

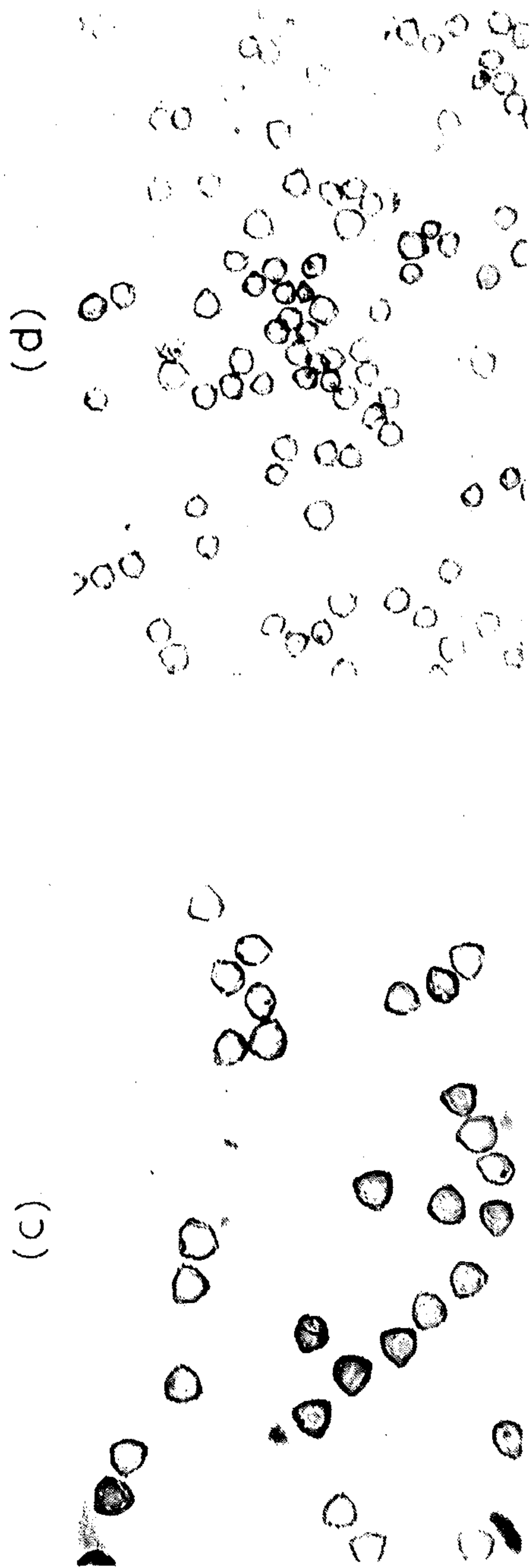
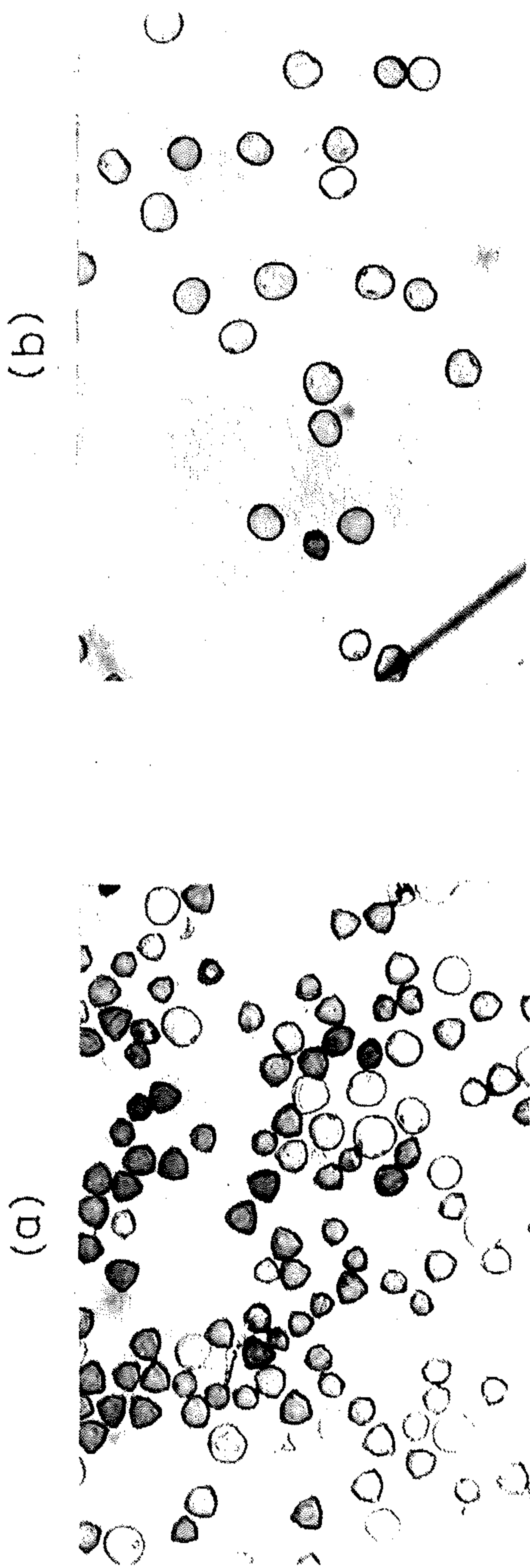
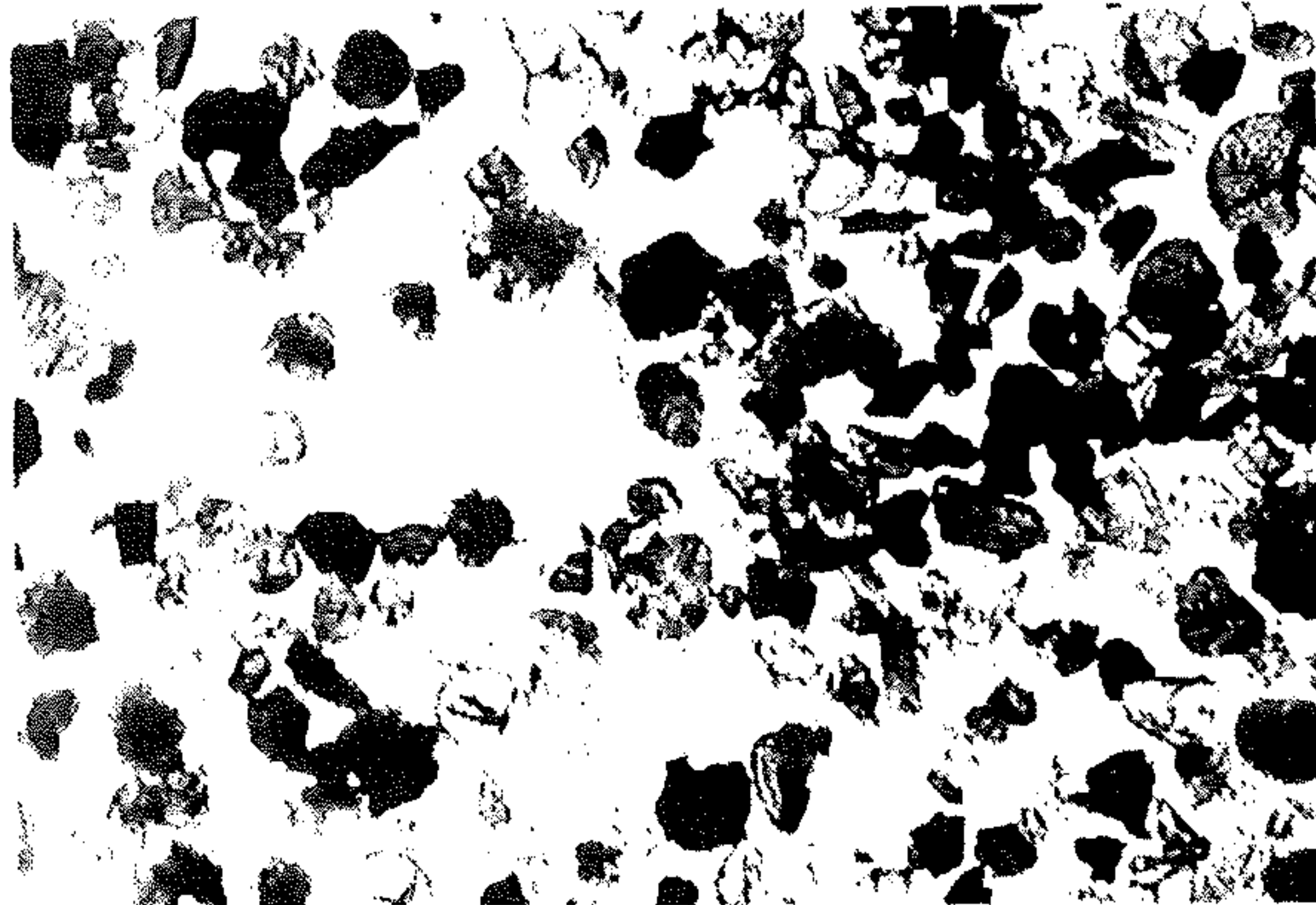


Plate 1 The separation of a mixture of three modern pollen types.

(a)



(b)



(c)

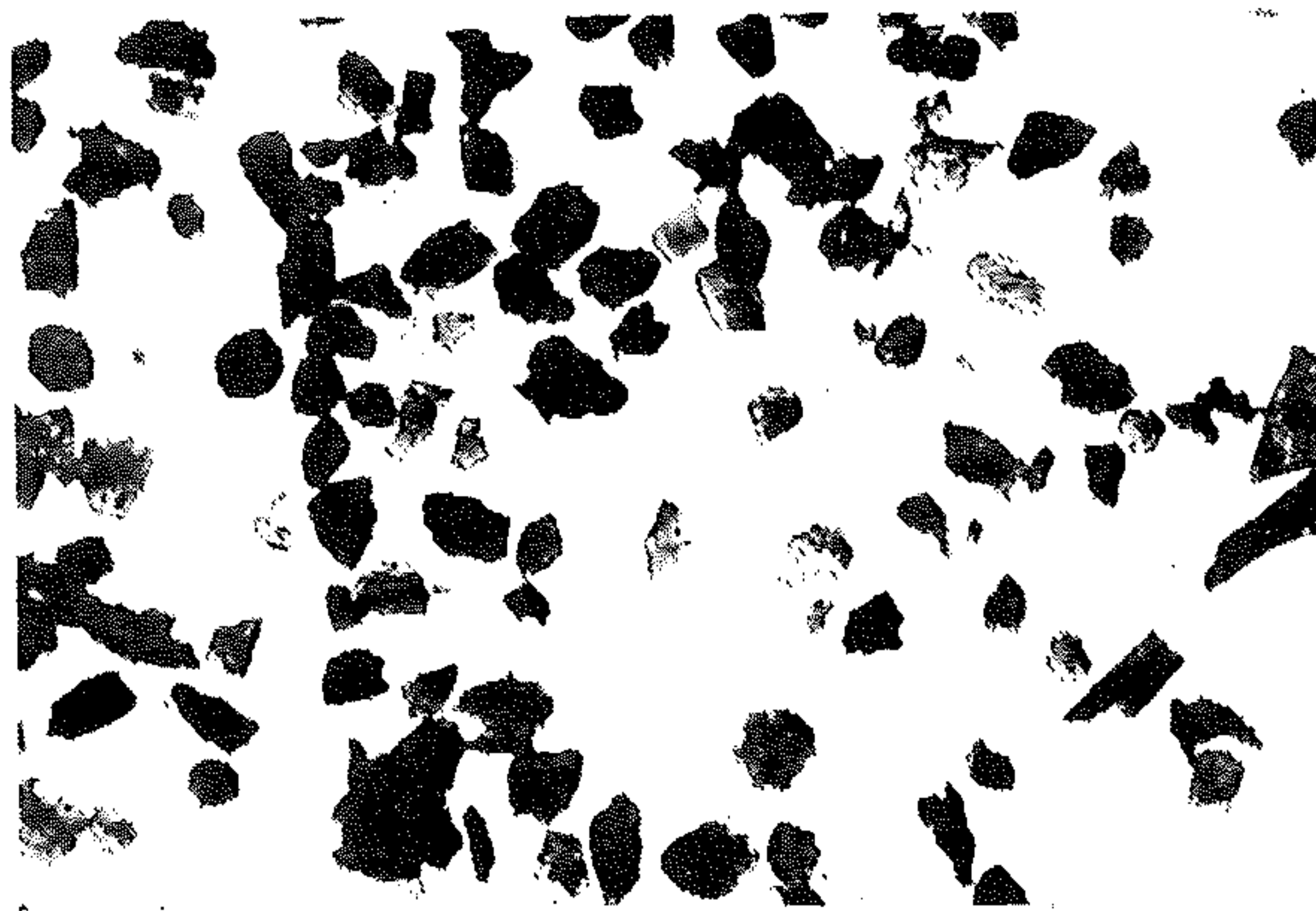


Plate 2 The purification of Palaeocene palynomorphs.

The density range covered by the five species is 0.0815 gcm^{-3} and from a knowledge of the relative positions of species in density gradients it is concluded that the density range of all the species so far dealt with is in excess of 0.1 gcm^{-3} . It appears that individual species may span a range of up to c. 0.05 gcm^{-3} on a density gradient though it is not clear what, if any, of this variation may be explained by the conditions of gradient loading.

- e.) Exine densities measured using KI solution density gradients are consistently higher than those measured using propanone/1,2-dibromoethane gradients. This is to be expected as different gradient materials are known to give different results (Williams and Wilson, 1981).
- f.) Certain chemical treatments have been shown to affect exine density. Sodium chlorate oxidation and treatment (oxidation) with hot 2-aminoethanol both produce a considerable increase in exine density.
- g.) With sediment samples prepared by conventional methods, including a chemical (chlorine) oxidation, it has been possible to remove the bulk of the organic detritus on a potassium iodide solution density gradient. Much of the detritus (which is probably mainly lignaceous) sediments completely leaving a purified pollen suspension. Plates 2.a,b, and c illustrate this phenomenon. 2.a shows the organic matter extracted from a Paleocene soft rock by conventional means; the sample is rich in pollen and spores but also contains an abundance of detrital organic fragments which obscure palynomorphs and frustrate identification and rapid counting. 2.b is the suspension recovered from a density gradient and is almost pure, the higher density organic detritus having been removed (2.c). It is possible to perform the cleaning operation without significantly changing the species proportions, as judged by chi-square test (Mosimann, 1965).
- h.) Fossil pollen types freed from a matrix by the method described in (g) above have been shown to exhibit some tendency to occupy characteristic equilibrium levels in a density gradient, relative to each other. As an illustration of this Fig. 6 shows the results of analysis of a density gradient through which a Flandrian organic lake mud preparation has been centrifuged and Fig. 7 the same for a Flandrian peat. The similar relative positioning of the *Corylus*, *Alnus*, *Ulmus*, *Tilia*, *Quercus* and *Betula* peaks is apparent. Plates 3a to d refer to the lake mud analysis (Fig. 6). Plate 3.a is a photomicrograph of the sample which was applied to the top of the gradient: a mixture of fossil pollen types and organic debris.

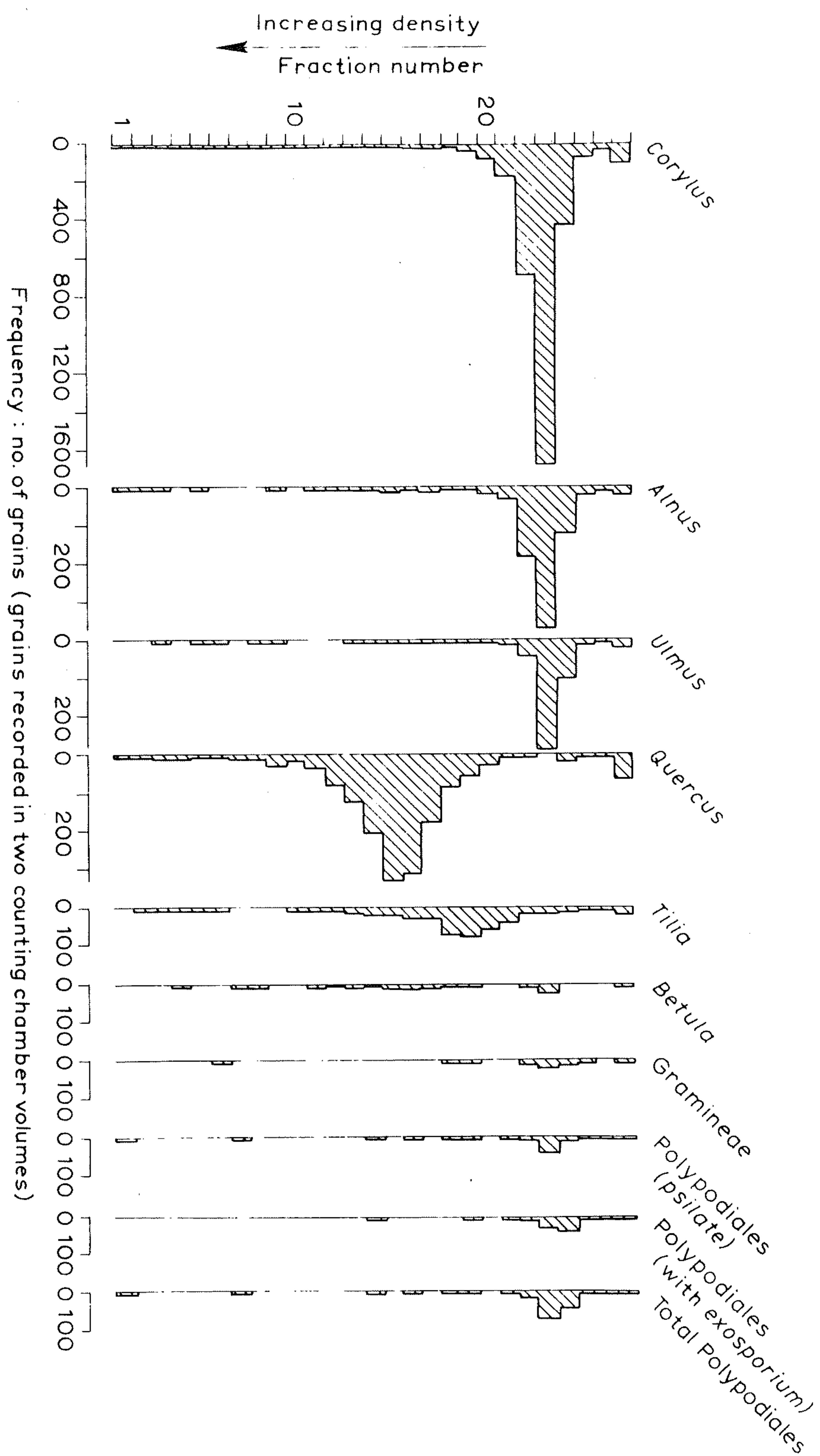


Figure 6 Distribution of pollen types from a Flandrian lake mud in a density gradient.

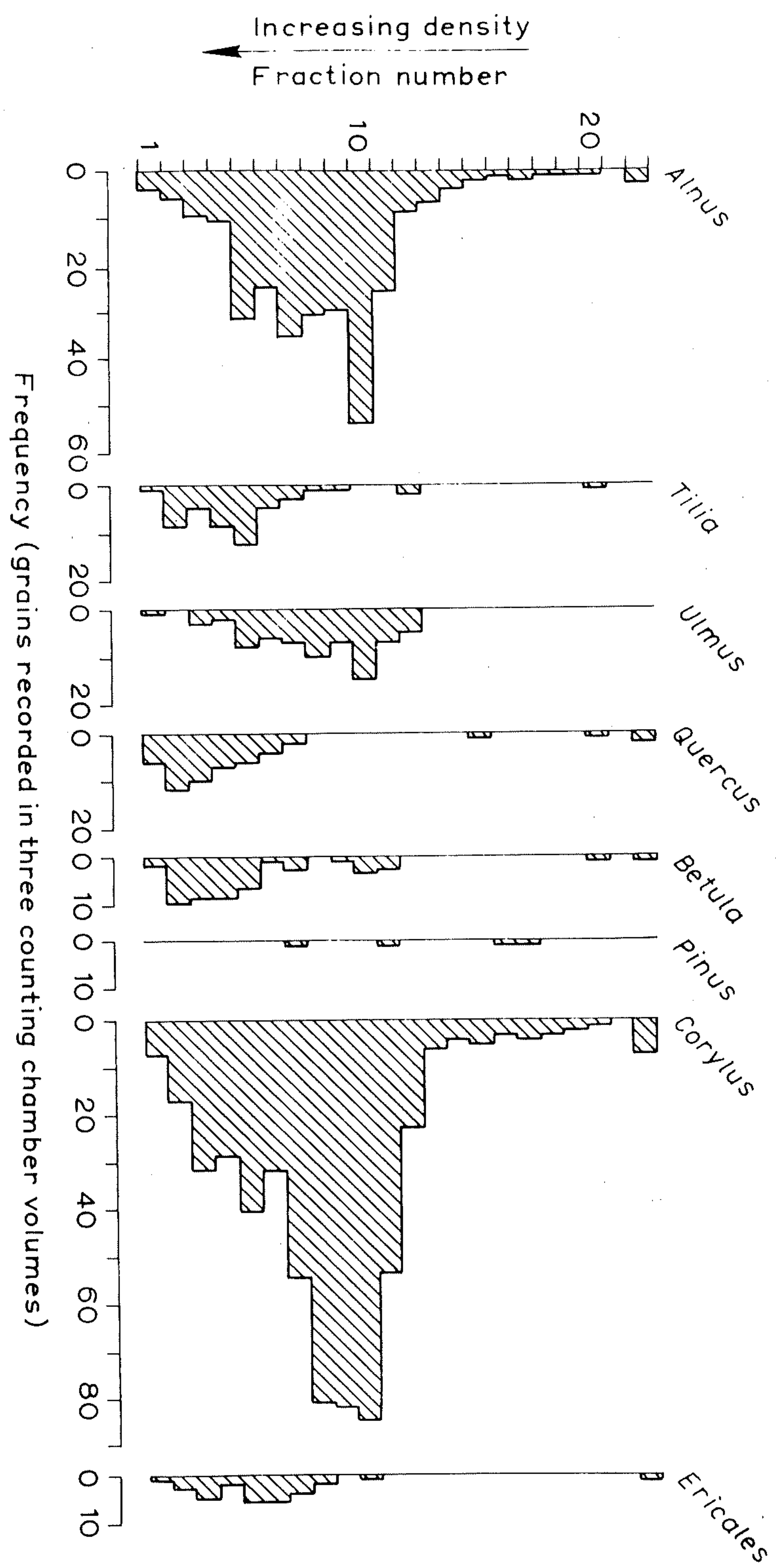


Figure 7 Distribution of pollen types from a Flandrian peat in a density gradient.

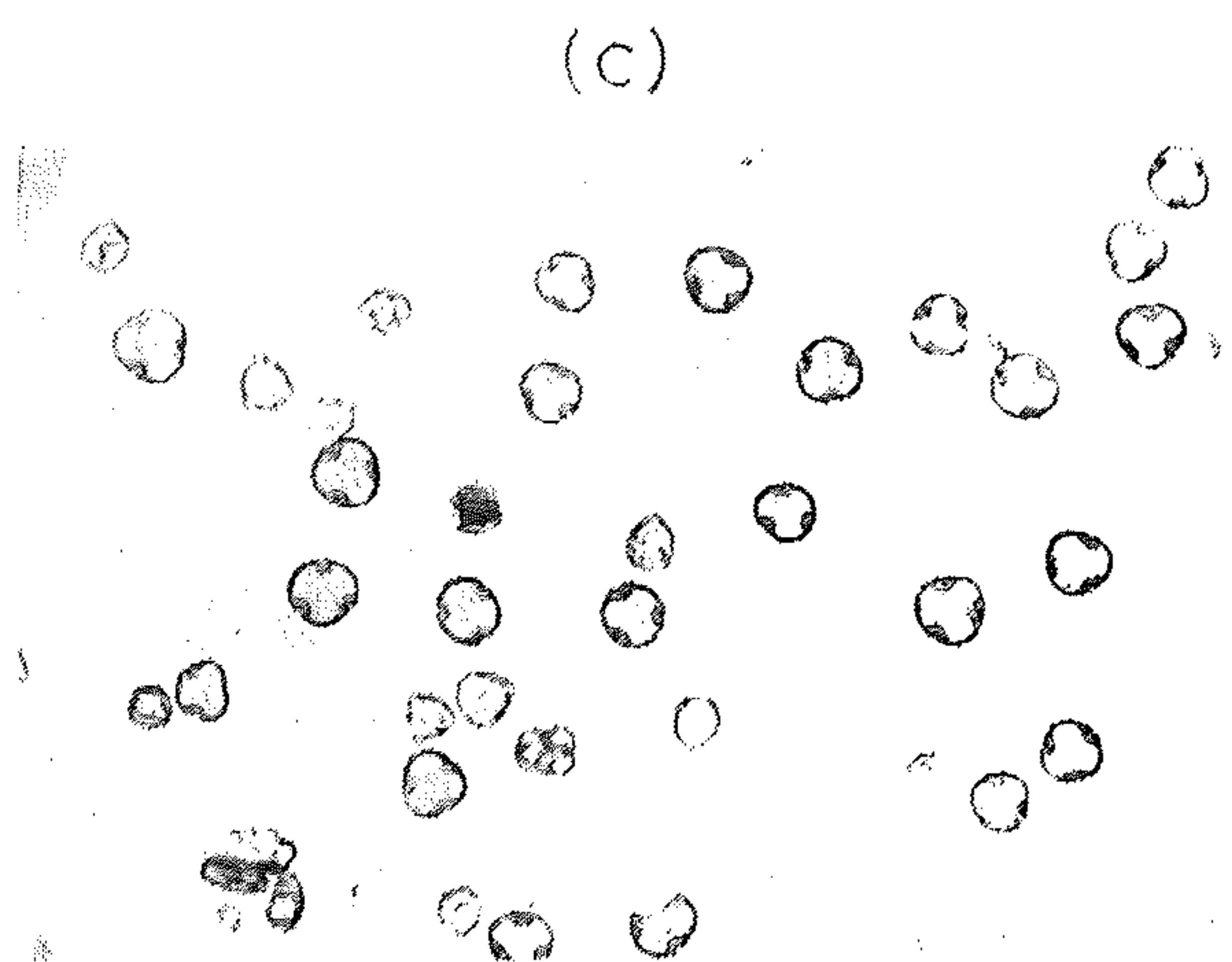
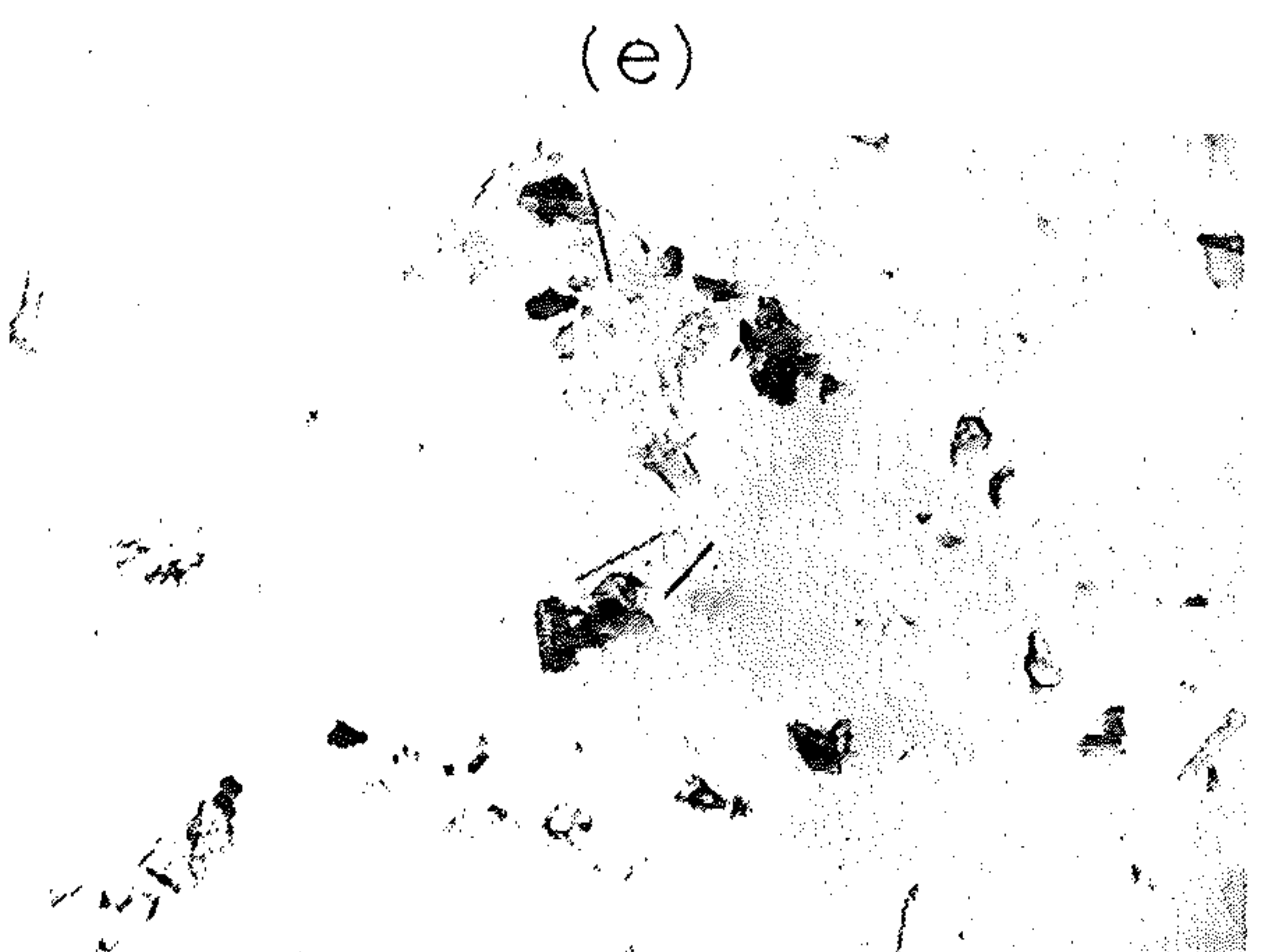
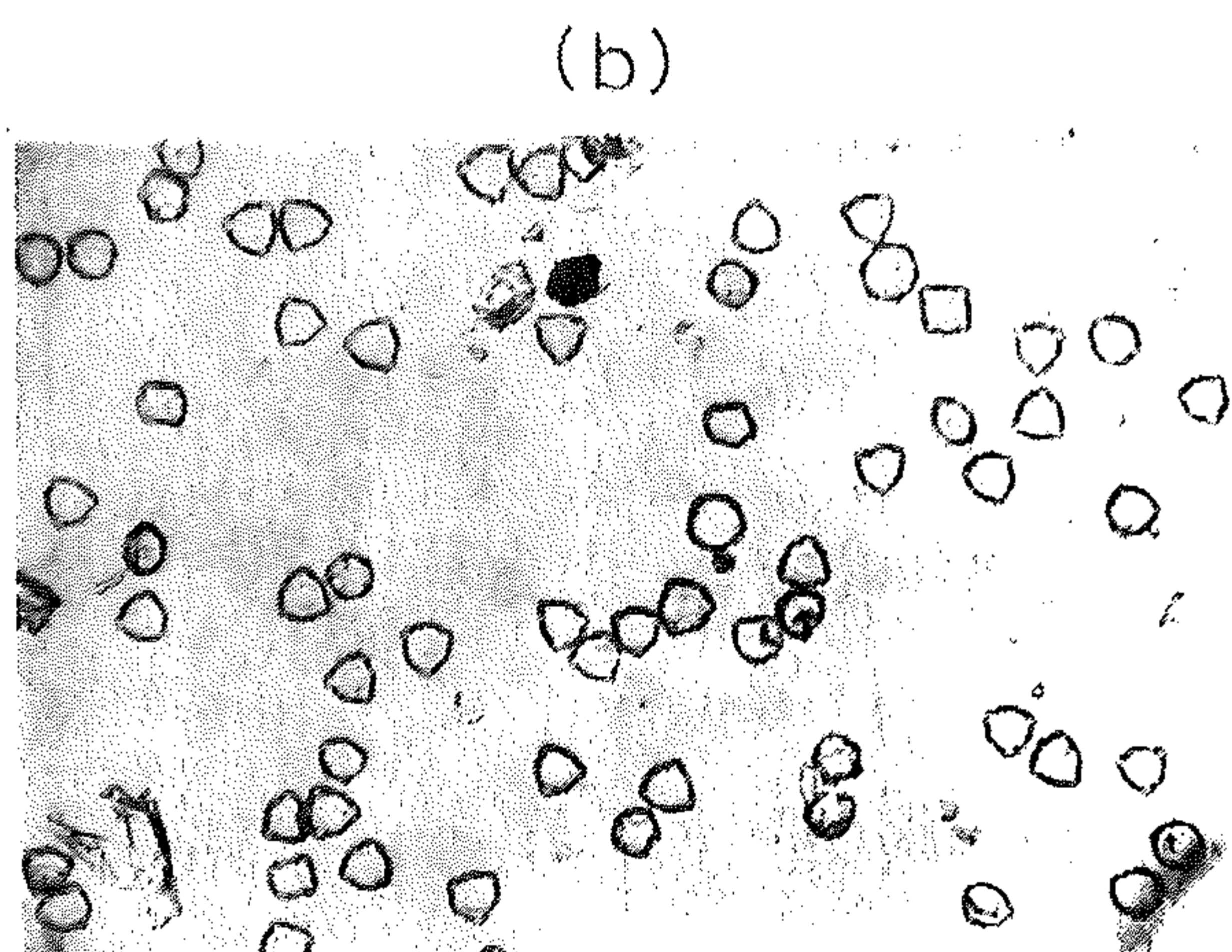
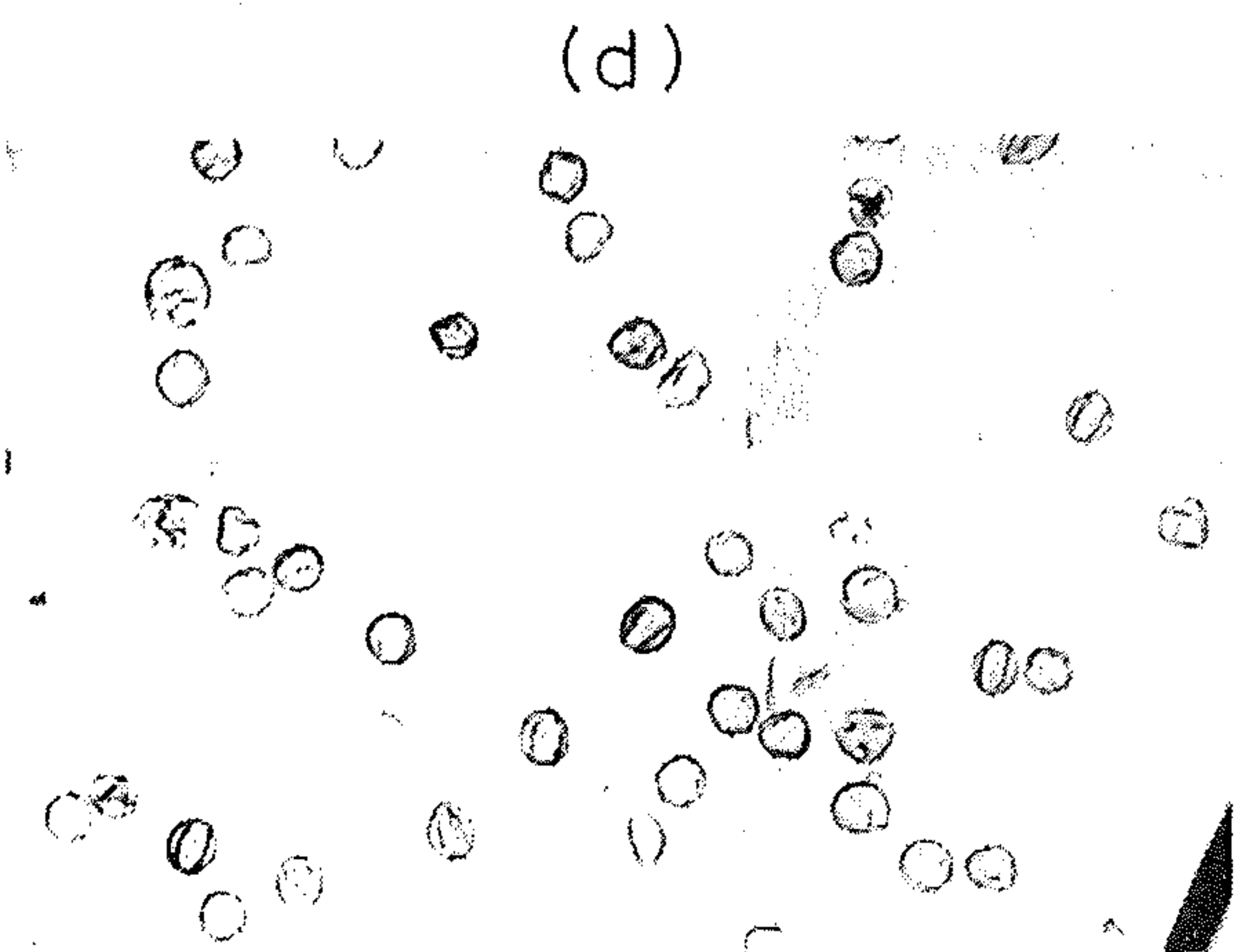
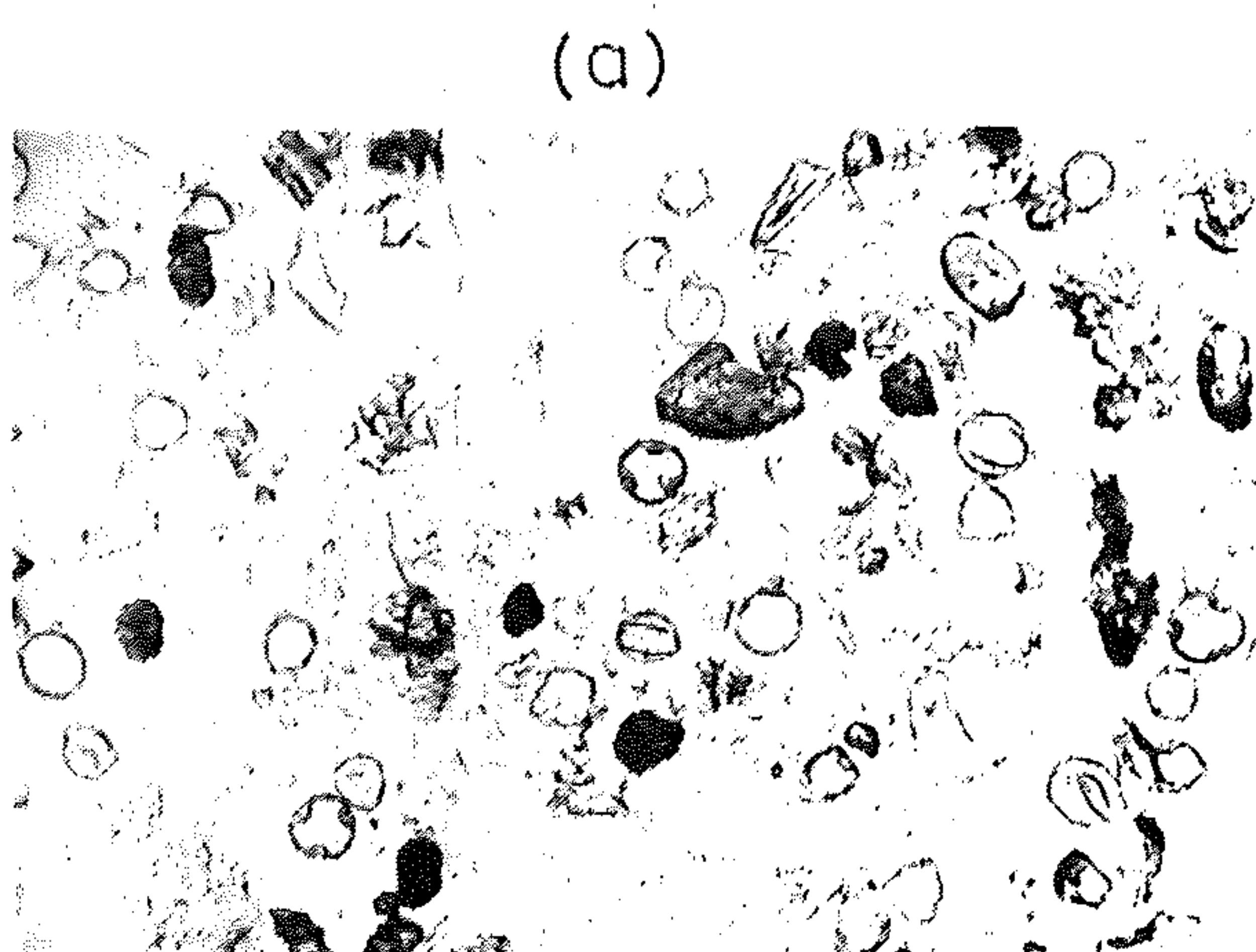


Plate 3 The purification and separation of Flandrian fossil pollen types.



Plate 4 Isolation of Flandrian fossil Polypodium spores.

Plates 3.b, c and d show the pollen assemblages recovered from fractions 23, 19 and 15 of the gradient corresponding to peaks in concentration of *Corylus/Alnus/Ulmus*, *Tilia* and *Quercus* respectively: much of the detrital organic material has been removed. The sedimented fraction is shown in plate 1.e: it is thought to consist chiefly of lignaceous material with some mineral matter. Plate 4 shows fossil *Polypodium* spores isolated from a similar lake mud; these tend to be of low density and form one of the highest bands in a density gradient.

CONCLUSIONS

- a.) The sensitivity of the exine to chemical treatment and the inherent variation in exine density within pollen types make it unlikely that anything other than major groups of fossil pollen species may be recognised specifically by their absolute positions in a density gradient, at least on anything approaching a routine basis. However, the isolation of a monospecific subsample from a mixed, fossil pollen flora (Plates 3 and 4), the first time that this has been achieved, may be considered significant.
- b.) There is a distinct possibility that fossil pollen preparations could be thoroughly cleaned of extraneous organic matter by a density gradient technique without affecting composition, even to the standard which might be required for computer based identification methods.
- c.) The technique may prove to have some application in exine chemical and structural investigations, particularly those based upon chemical dissection of the exine. Rowley *et al.* (1981) and Rowley and Prijanto (1977) contain examples of the sort of study to which the technique may be able to contribute.

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